Novel Mechanism of Enzymatic Hydrolysis Involving Cyanoalanine Intermediate Revealed by Mass Spectrometric Monitoring of an Enzyme Reaction (MOLECULAR BIOFUNCTION-Molecular Microbial Science)

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Nobuyoshi Esaki, Tatsuo Kurihara, Yong-Fu Li and Susumu Ichiyama

L-2-Haloacid dehalogenase from *Pseudomonas* sp. YL catalyzes the hydrolytic dehalogenation of L-2-haloalkanoic acids to produce the corresponding D-2-hydroxyalkanoic acids. Asp10 of the enzyme functions as a catalytic nucleophile: the residue attacks the α-carbon of the substrate to form an ester intermediate, which is subsequently hydrolyzed to release the product. Surprisingly, replacement of Asp10 by Asn did not completely inactivate the enzyme. We found that Asn10 of the D10N mutant enzyme is spontaneously deamidated to yield Asp, though slowly, causing increasing activity of the D10N preparation. We also revealed by mass spectrometric monitoring of the enzyme reaction that the D10N mutant enzyme itself catalyzes the hydrolytic dehalogenation: Asn10 attacks the substrate to form an imidate, and a proton and D-lactic acid are eliminated to produce a nitrile (a β-cyanoalanine residue), followed by hydrolysis to reproduce Asn10. This is the first report of the function of Asn to catalyze nucleophilic substitution through its dynamic structural change that includes conversion to a β-cyanoalanine residue as an intermediate structure.

Keywords: 2-Haloacid dehalogenase/ Ion-spray mass spectrometry/ Cyanoalanine residue

L-2-Haloacid dehalogenase from *Pseudomonas* sp. YL (L-DEX YL) catalyzes the hydrolytic dehalogenation of L-2-haloalkanoic acids, producing the corresponding D-2-hydroxyalkanoic acids. The enzyme is involved in biodegradation of xenobiotic compounds such as a herbicide, 2,2-dichloropropionic acid. The enzyme is also useful as an industrial biocatalyst for the synthesis of optically active hydroxyalkanoic acids. We have studied the reaction mechanism of L-DEX YL in detail, and revealed that the reaction proceeds as follows [1, 2]. Asp10 of the enzyme nucleophilically attacks the α-carbon of the substrate to release a halide ion and produce an ester intermediate, which is subsequently hydrolyzed to release a D-2-hydroxyalkanoic acid and regenerate Asp10. We performed mutagenesis studies on L-DEX YL, and surprisingly found that replacement of Asp10 by Asn did not completely inactivate the enzyme, whereas replacement by Ala, Gly, Ser, or Glu resulted in total inactivation [3].

MOLECULAR BIOFUNCTION — Molecular Microbial Science —

Scope of research

Structure and function of biocatalysts, in particular, pyridoxal enzymes and enzymes acting on xenobiotic compounds, are studied to elucidate the dynamic aspects of the fine mechanism for their catalysis in the light of recent advances in gene technology, protein engineering and crystallography. In addition, the metabolism and biofunction of sulfur, selenium, and some other trace elements are investigated. Development and application of new biomolecular functions of microorganisms are also studied to open the door to new fields of biotechnology. For example, molecular structures and functions of psychrophilic enzymes and their application are under investigation.

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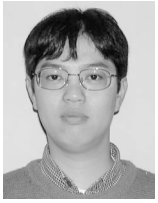

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In the present study, we analyzed the mechanism of dehalogenation catalyzed by the L-DEX YL D10N preparation by ion-spray mass spectrometry [4].

We found that the activity of the D10N preparation increased in a time- and temperature-dependent manner. When determined immediately after purification of the enzyme, it showed about 1% activity of the wild-type enzyme activity, whereas it showed 26% activity of the wild-type enzyme activity when stored at room temperature for about a month. Amino acid sequencing of the enzyme preparation showing 26% activity revealed the occurrence of Asp at the position of Asn10. These results indicate that the side chain amide of Asn10 is slowly deamidated to produce carboxylate.

We next examined whether the D10N mutant enzyme itself is involved in the dehalogenation by ion-spray mass spectrometry. Although the D10N preparation is contaminated by the wild-type enzyme produced by the spontaneous deamidation of Asn10 as described above, the wild-type enzyme content in the fresh preparation is considered to be less than 1% of the total enzyme molecules judging from the enzyme activity of the preparation. Thus it is possible to monitor the structural change of D10N itself by mass spectrometry, because the small amount of the wild-type enzyme does not interfere with the mass spectra of D10N, which is present abundantly. We first confirmed that the control enzyme not incubated with the substrate showed a peak at 26,180 Da (M), which is virtually identical to the molecular mass (26,178 Da) predicted from the primary structure of the D10N enzyme, and then incubated the enzyme preparation with L-2-chloropropionate as a substrate to monitor the structural change of the enzyme. After 10 s of the incubation, the original peak at around 26,178 Da disappeared, and new peaks appeared at 26,253 Da (M+73) and 26,162 Da (M-18). Over a period from 10 s to 1 min, the relative abundance of the M+73 species decreased, and the M-18 species increased. From 20 s to 40 min, the enzyme occurred predominantly as the M-18 species. The original peak (M) reappeared and increased over a period of 30-60 min, and became predominant by 60 min.

We determined the N-terminal amino acid sequence of the M+73 species, and found that the Asn10 was modified. To analyze the modification at position 10 by mass spectrometry, we constructed D10N/L11K double mutant enzyme: lysyl-endopeptidase treatment of this enzyme produces a short peptide fragment containing Asn10, which is small enough for determination of accurate molecular mass. We confirmed that the mutation at position 11 did not alter the reactivity of the D10N enzyme as judged by structural change of the enzyme revealed by mass spectrometry. D10N/L11K incubated with L-2-chloropropionate was digested with lysyl endopeptidase, and the resultant peptide containing Asn10 was analyzed by tandem MS/MS. We found that the modification causing the 73-Da increase occurred at Asn10, and revealed that an imidate structure was produced as a reaction intermediate (Fig. 1): we found that the M+73 species was artificially produced by hydrolysis of the imidate (M+72) under a low-pH condition employed in the present experiment to terminate the enzyme reaction.

We next analyzed the structure of the M-18 species. The M-18 species derived from D10N/L11K was digested with lysyl endopeptidase, and the peptide containing Asn10 was analyzed by tandem MS/MS. We found that Asn10 was specifically modified in such a way as its molecular mass becomes 18-Da lower than the original value. The most probable structure formed at residue number 10 is a β-cyanoalanine residue as shown in Fig. 1.

To examine if the final step of the reaction is the reconversion of β-cyanoalanine residue into Asn as shown in Fig. 1, D10N/L11K was incubated in $H_2{}^{18}O$ in the presence of L-2-chloropropionate for 60 min. After the incubation, we analyzed the molecular mass of the peptide containing Asn10, and found that an ^{18}O atom was incorporated in Asn10. This result indicates that the nitrile undergoes the nucleophilic attack of a water molecule to produce the side chain amide of Asn10.

In colclusion, we revealed a unique structural change of L-DEX YL D10N that occurs through the mechanism shown in Fig. 1: Asn10 nucleophilically attacks the substrate to form the imidate, and a proton and D-lactic acid are eliminated to produce the nitrile. This is the first report showing that Asn functions as a catalytic nucleophile in enzymatic hydrolysis where β-cyanoalanine residue is produced as a reaction intermediate.

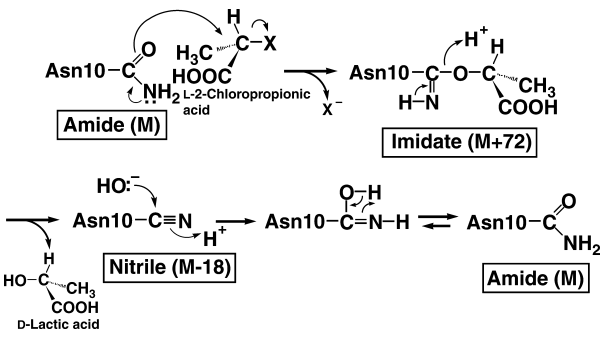

Figure 1. Structural change of D10N involving an imidate and a nitrile as reaction intermediates.

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